

Effects of Organ Culture and Optisol-GS Storage on Structural Integrity, Phenotypes, and Apoptosis in Cultured Corneal Epithelium

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PURPOSE. A previous report has described the use of eye bank storage of cultured human limbal epithelial cells (HLECs) to provide a reliable source of tissue for treating limbal stem cell deficiency. In the present study, conventional organ culture (OC) storage and Optisol-GS (Bausch & Lomb, Irvine, CA) storage of cultured HLECs were compared.

METHODS. Three-week HLEC cultures were either organ cultured at 31°C or 23°C or stored in Optisol-GS at 5°C in a closed container for 1 week. Morphology was studied by light microscopy and transmission electron microscopy, and phenotypic characterization was assessed by immunohistochemistry. Apoptosis was evaluated by real-time RT-PCR microarray analysis, caspase-3 immunohistochemistry, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL).

RESULTS. The ultrastructure was preserved at 23°C, while storage at 31°C and 5°C was associated with enlarged intercellular spaces, separation of desmosomes, and detachment of epithelial cells. Cultured HLECs remained undifferentiated in all storage conditions. The expression of the antiapoptotic gene *BCL2* was prominently upregulated in storage at 23°C and 5°C. Downregulation of *BCL2A1*, *BIRC1*, and *TNF* and upregulation of *CARD6* in 23°C and 5°C storage conditions suggests a reduction in nuclear factor-κB activity. No significant increase in cleaved caspase-3 and TUNEL staining was observed in response to eye bank storage, and the labeling indices of cleaved caspase-3 (range, 0.0%–4.7%) and TUNEL (range, 0.0%–7.8%) were low.

CONCLUSIONS. These data indicate that OC storage of cultured HLECs at ambient temperature is superior to OC storage at 31°C and Optisol-GS storage at 5°C and that apoptosis is minimal after eye bank storage of cultured HLECs. (*Invest Oph-*

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Transplantation of ex vivo expanded human limbal epithelial cells (HLECs) is a therapy for limbal stem cell deficiency (LSCD).^{1–6} HLECs may be cultured ex vivo in a variety of expansion protocols, including limbal explant culture,^{7–10} cell suspension culture,^{1,7,11,12} culture on intact^{9,10,13,14} or epithelially denuded^{7,12–16} amniotic membranes (AMs) or other cell culture surfaces,^{1,7,17–21} cocultivation with lethally irradiated 3T3 fibroblasts,^{1,3,8,11} and air-lifting.^{4,22} An alternative approach in treating LSCD has been the use of autologous oral mucosal epithelial sheets.^{23–26} Although the protocols have shown good clinical outcomes, limbal epithelial stem cell therapy still faces challenges regarding surgery logistics, tissue sterility, tissue transportation, and availability of tissue. The timing of surgery may be complicated, as the engineering of multilayered epithelia requires culture periods of 3 to 4 weeks, and the tissue cultures are susceptible to microbial contamination during the setup of the cultures, medium change, and transportation to the operating theater. The clinical application of limbal epithelial stem cell therapy is currently limited to ophthalmology departments with a knowledge of tissue engineering and laboratory facilities available for the procedures.

Our laboratory was the first to report a method of short-term eye bank storage of cultured HLECs that may be beneficial in limbal epithelial stem cell therapy.²⁷ In our study, 3-week HLEC cultures were transferred from the incubator to a glass container with organ culture (OC) medium and stored for 1 week at 23°C, during which they maintained the original multilayered structure and undifferentiated phenotype (Fig. 1). The experimental design of this method has several advantages. First, the maintenance of the limbal phenotype offers flexibility in scheduling the transplantation. Second, tissue storage allows time to perform microbiologic testing of the storage medium, which may enhance the safety of transplantation of ex vivo expanded HLECs. Third, the closed system enables tissue to be transported from the laboratory to the operating theater and between eye banks to increase the availability of tissue. Finally, storage at room temperature eliminates the need for heating cabinets.

The novel preservation method raises fundamental eye bank questions regarding the optimal temperature and medium for the storage of cultured HLECs. Residual corneoscleral donor rims after penetrating keratoplasty, which are a source of HLECs for the engineering of cultured corneal epithelium,^{28–31} are generally stored in OC medium³² at temperatures between 31°C and 37°C³³, or in Optisol-GS³⁴ (Bausch & Lomb, Irvine, CA) at 4°C. Furthermore, storage of limbal epithelium in Optisol-GS has been shown to produce a basal layer cell viability of 95% after 6 days.³⁵

We hypothesized that OC storage at 31°C, which is the preferred temperature in 26 of 43 European Eye Banks,³³ and Optisol-GS hypothermic storage may preserve the characteris-

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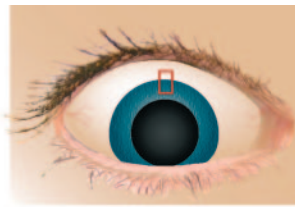
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A. Removal of limbal tissue from the eye

A limbal explant is excised from the healthy eye (for autologous transplantation) or a cadaver eye (for allogenic transplantation).

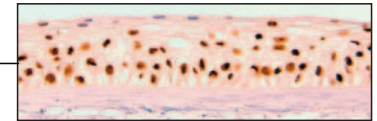


B. 3-week HLEC cultures

HLEC are cultured for three weeks on a human amniotic membrane that is fastened using a suture to the polyester membrane of a culture plate insert.

Amniotic membrane

Limbal explant



Cultured corneal epithelium prior to storage

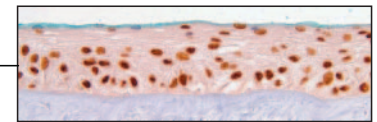
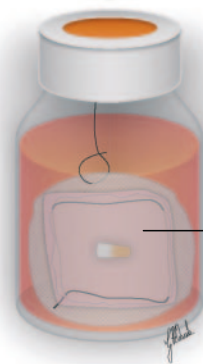
C. 1-week eye bank storage of cultured HLEC

The polyester mesh membrane with the cultured HLEC attached, is stored for one week at 23°C in organ culture medium consisting of Dulbecco's modified Eagle's medium with 7.5% sodium bicarbonate, 8% fetal bovine serum, 50 µg/mL gentamicin, 100 µg/mL vancomycin, and 2.5 µg/mL amphotericin B.

Benefits

1. Flexibility in scheduling transplantation
2. Time to perform microbiological testing
3. Safe tissue transportation

Polyester membrane



Cultured corneal epithelium following storage

FIGURE 1. Eye bank storage of cultured HLECs.

tics of cultured HLECs. Accordingly, we compared these conventional storage methods with the novel storage method. Moreover, because cell death due to apoptosis has been reported in human corneal epithelium after OC culture storage³⁶ and hypothermic storage,^{37,38} we studied expression of apoptosis-regulating genes and examined apoptosis markers in cultured HLECs after eye bank storage.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM), HEPES-buffered DMEM containing sodium bicarbonate and Ham's F12 (1:1), Hanks' balanced salt solution, fetal bovine serum (FBS), insulin-transferrin-selenium medium supplement, human epidermal growth factor, dimethyl sulfoxide, hydrocortisone, gentamicin, amphotericin B, and rabbit polyclonal anti-connexin 43 antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Dispase II was obtained from Roche Diagnostics (Basel, Switzerland) and cholera toxin A subunit from Biomol (Exeter, UK). 6-0 C-2 monofilament sutures (Ethicon Ethilon) from Johnson & Johnson (New Brunswick, NJ), culture plate inserts (Netwell) from Costar Corning (Corning, NY), vancomycin from Abbott Laboratories (Abbott Park, IL), Optisol-GS from Bausch & Lomb (Irvine, CA), and glass containers from OneMed (Vantaa, Finland). Mouse anti-p63 antibody (clone 4A4), mouse anti-CK19 antibody (clone RCK108), and mouse anti-Ki67 antibody (clone MIB-1) were obtained from Dako (Glostrup, Denmark), mouse anti-vimentin anti-

body (clone VIM 3B4) from Ventana Medical Systems (Tucson, AZ), and mouse anti-CK3 antibody (clone AE5) from ImmQuest (Seamer, UK). The following were obtained from Novocastra Laboratories Ltd. (Newcastle-upon-Tyne, UK): mouse anti-CK5 antibody (clone XM26), mouse anti-CK14 antibody (clone LL02), mouse anti-E-cadherin antibody (clone NCH-38), and mouse anti-integrin β 1 antibody (clone 7F10). Rabbit polyclonal anti-caspase-3 antibody was from Cell Signaling Technology (Danvers, MA). Epon was purchased from Electron Microscopy Sciences (Hatfield, PA). An RNA isolation kit (ArrayGrade FFPE), PCR array (RT² Apoptosis Profiler; cat. no. APHS-012), first-strand synthesis kit (RT² PCR array True Labeling Picoamp kit), and PCR master mix (RT² Real-Time SYBR Green PA-012) were obtained from SuperArray Bioscience (Frederick, MD). A 384-well block (7900HT) was purchased from Applied Biosystems (Foster City, CA), and a colorimetric TUNEL system kit was obtained from Promega Corp. (Madison, WI).

Human Tissue Preparation

Human tissue was handled according to the Declaration of Helsinki. Corneoscleral tissues were obtained from the Norwegian Corneal Eye Bank (Oslo, Norway) after the central corneal button had been used for corneal transplantation. The experiment was conducted on four pairs of corneoscleral rims from the same human donors as in our previous study,²⁷ and the study of the four experimental groups (3-week HLEC culture and storage at 31°C, 23°C, and 5°C) was run concurrently. The

TABLE 1. Semiquantitative Immunohistochemical Localization of Ocular Surface Markers in Cultured HLECs in Three Storage Conditions

Antigen	Antibody Dilution	3-Week HLEC Culture at 37°C*		1-Week OC Storage at 31°C†		1-Week OC Storage at 23°C*		1-Week Optisol-GS Storage at 5°C	
		B	SB	B	SB	B	SB	B	SB
p63	1:25	+++	++	+++	+++	+++	+++	+++	+++
K19	1:200	++	++	++	++	++	++	+++	+++
Vimentin	RTU	+++	++	+++	++	+++	++	+++	++
Ki67	1:75	+	0/+	+	+	0/+	0	0	0/+
K3	1:500	0	0	0	0	0	0	0	0
K5	1:600	+++	+++	+++	+++	+++	+++	+++	+++
K14	1:80	+++	+++	++	++	+++	+++	++	+++
Cx43	1:500	++	++	++	++	+	+	+	+
E-Cadherin	1:25	+	++	+	++	+	++	++	+
Integrin β 1	1:10	++	+	+	0	++	0	+++	++

The immunoreactivity was graded as 0 (undetectable), + (weak positivity of >50% cells), ++ (intermediate positivity of >50% cells), and +++ (strong positivity of >50% cells). All scores were assigned at a magnification of $\times 400$ by two independent experienced investigators blinded to the origin of the samples. OC, organ culture; B, basal layer; SB, suprabasal layer; RTU, ready to use.

* The results for 3-week HLEC culture at 37°C and 1-week organ culture storage at 23°C have been reported.²⁷

† One of eight samples was excluded from the analysis due to extensive ingrowth of fibroblasts.

limbal tissue was prepared as previously reported by Meller et al.¹⁰ The tissue was rinsed three times with DMEM containing 50 μ g/mL gentamicin and 1.25 μ g/mL amphotericin B. After careful elimination of excessive sclera, conjunctiva, iris, and corneal endothelium, the remaining tissue was placed in a culture dish and exposed for 10 minutes to Dispase II (1.2 U/mL) in Mg^{2+} and Ca^{2+} free Hanks' balanced salt solution at 37°C under humidified 5% carbon dioxide. After one rinse with DMEM containing 10% FBS, every corneoscleral rim was divided into 12 limbal explants, which were equally distributed among the four experimental groups.

Human Limbal Explant Cultures on Intact Amniotic Membranes

Human AMs were preserved in accordance with a method previously reported by Lee and Tseng³⁹ and according to the Declaration of Helsinki. After they were thawed at room temperature, AMs with the epithelium intact and facing up was fastened to the polyester membrane of a culture plate insert with 6-0 monofilament sutures (Ethicon Ethilon; Johnson & Johnson) (Fig. 1), as previously reported.²⁷ Limbal explant cultures were prepared as described elsewhere.¹⁰ On the center of each AM insert, a human limbal explant was cultured in supplemented hormonal epithelial medium made of HEPES-buffered

DMEM containing sodium bicarbonate and Ham's F12 (1:1). The medium was supplemented with 5% FBS, 0.5% dimethyl sulfoxide, 2 ng/mL human EGF, 5 μ g/mL insulin, 5 μ g/mL transferrin, 5 ng/mL selenium, 3 ng/mL hydrocortisone, 30 ng/mL cholera toxin, 50 μ g/mL gentamicin, and 1.25 μ g/mL amphotericin B. The cultures were incubated for 3 weeks at 37°C in an atmosphere of humidified 5% carbon dioxide and 95% air, and the medium was changed every 2 to 3 days.

Eye Bank Storage of Cultured HLECs

The HLEC cultures ($n = 36$) were prepared for eye bank storage as previously reported,²⁷ and 3-week HLEC cultures ($n = 12$) served as control samples. The polyester mesh membrane with the cultured epithelium attached was released by using a steel blade and suspended in a sterilized 50-mL glass container with a 6-0 monofilament suture, which was tied to the edge of the polyester membrane and the rubber cap (Fig. 1). The cultured HLECs were stored for 1 week in either 50 mL organ culture medium containing Dulbecco's modified Eagle's medium with 7.5% sodium bicarbonate, 8% FBS, 50 μ g/mL gentamicin, 100 μ g/mL vancomycin, and 2.5 μ g/mL amphotericin B at 31°C ($n = 12$) or 23°C ($n = 12$), or in 50 mL Optisol-GS at 5°C ($n = 12$). The glass containers were each closed by a rubber cap to establish a closed tissue storage system.

3-week HLEC culture

1-week organ culture storage at 31°C

1-week Optisol-GS storage at 5°C

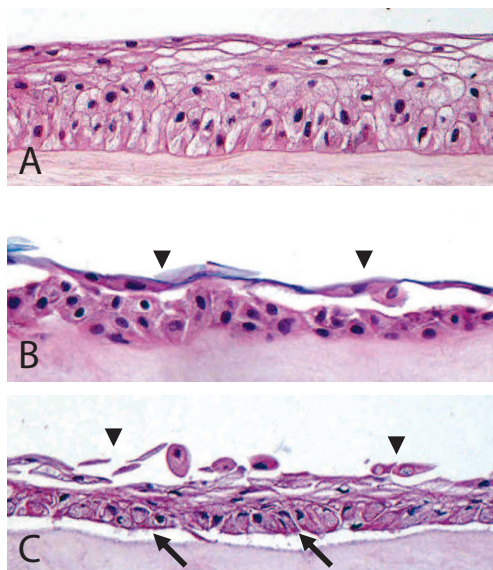


FIGURE 2. Sections stained with H&E in cultured human limbal epithelial cells after 3 weeks' culture (A) and 1 week's storage at 31°C (B) or 5°C (C). Arrowheads: detachment of epithelial cells; arrows: basal layer detachment from the amniotic membrane. Original magnification, $\times 400$.

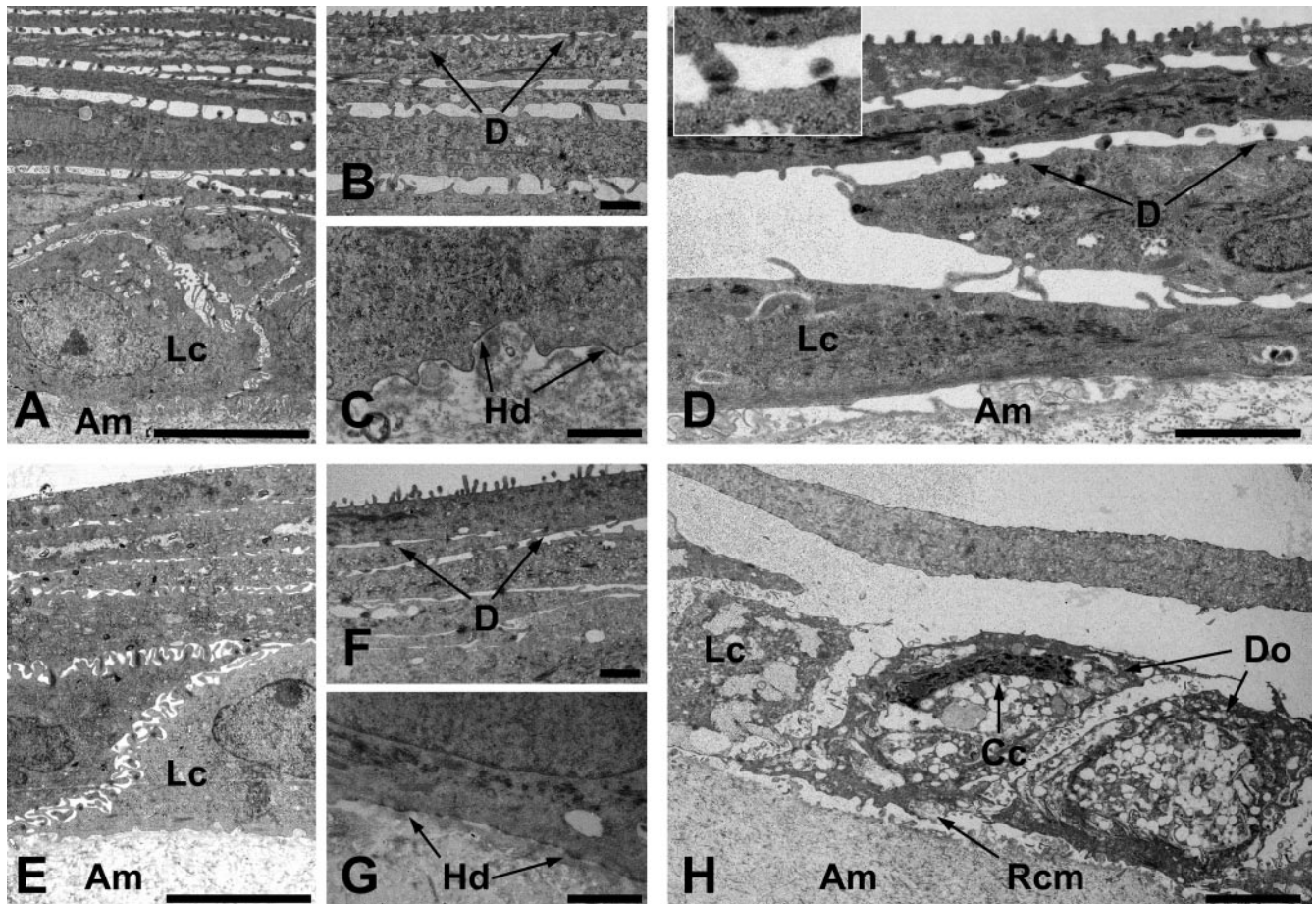


FIGURE 3. Transmission electron micrographs showing cultured human limbal epithelial cells after 3 weeks' culture and 1 week's storage at three different temperatures. (A) Three-week HLEC cultures demonstrated a multilayered epithelium with numerous intercellular desmosomes (B, arrows) and hemidesmosomes (C, arrows) promoting adhesion to the amniotic membrane. (D) In organ culture conditions at 31°C, dilated intercellular spaces, detachment of desmosome complexes (inset, arrows), and poor adhesion to the amniotic membrane were revealed. (E) The original epithelial structure was preserved after 1 week of organ culture storage at 23°C with numerous desmosomes (F, arrows) and hemidesmosomes (G, arrows). (H) Optisol-GS storage at 5°C induced dilated intercellular spaces, detachment of epithelial cells, detachment of the epithelia from the amniotic membrane, and an increase in the number of intracellular vacuoles. In addition, weak to moderate chromatin condensation (arrows), rupture of cell membranes (arrows), and dissolution of organelles (arrows) were regularly observed. Lc, limbal epithelial cell; Am, amniotic membrane; D, desmosomes; Hd, hemidesmosomes; Cc, chromatin condensation; Rcm, rupture of cell membranes; Do, dissolution of organelles. Scale bars: (A) 10 μ m; (B, C, F, G) 1 μ m; (D) 2 μ m; (E, H) 5 μ m.

Histology and Immunostaining

Eight cultures from each experimental group were fixed in neutral buffered 4% formaldehyde and embedded in paraffin. Serial sections of 5 μ m were routinely stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed with a panel of antibodies for markers of human ocular surface epithelia (Table 1). To visualize the immunoreactions, we used a standard peroxidase technique (DAB [3,3'-diaminobenzidine] detection kit) with an automated immunostaining system (model ES; Ventana Medical Systems, Tucson, AZ). Optimal antibody dilutions were determined by titration with the positive controls recommended by the manufacturers. A conventional immunohistochemical scoring system was used as previously reported.^{40,41} The immunoreactivity was graded as 0 (undetectable), + (weak positivity of >50% cells), ++ (intermediate positivity of >50% cells), or +++ (strong positivity of >50% cells). All scores were assigned at a magnification of $\times 400$ by two independent experienced investigators blinded to the origin of the samples.

Transmission Electron Microscopy

Four cultures from each experimental group were fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer adjusted to pH 7.4, postfixed in 1%

osmium tetroxide, and dehydrated through a graded series of ethanol up to 100%. The tissue blocks were immersed in propylene oxide twice for 20 minutes and embedded in Epon. Ultrathin sections were cut on a microtome (Ultracut Ultramicrotome UCT; Leica, Wetzlar, Germany) and examined with a transmission electron microscope (model CM120; Philips, Amsterdam, The Netherlands).

Real-Time Quantitative RT-PCR

RNA was isolated from the formalin-fixed paraffin-embedded (FFPE) tissue applying an RNA isolation kit (ArrayGrade FFPE), according to the manufacturer's protocol (SuperArray Bioscience). Three biological replicates were randomly selected from each experimental group. The human apoptosis PCR array (RT² Profiler) was used to analyze mRNA levels of 84 key genes involved in apoptosis, in a 384-well format, according to the manufacturer's instructions (SuperArray Bioscience). In brief, approximately 30 to 40 ng RNA was first amplified by using a modified version of a kit (True Labeling Picoamp; Superarray Bioscience). First-strand cDNA was synthesized with 400 ng of amplified cRNA by using a PCR array first strand-synthesis kit (C-02; Superarray Bioscience). This kit uses reverse transcriptase (PowerScript; Superarray Bioscience) and a combination of random primers and oligo dT

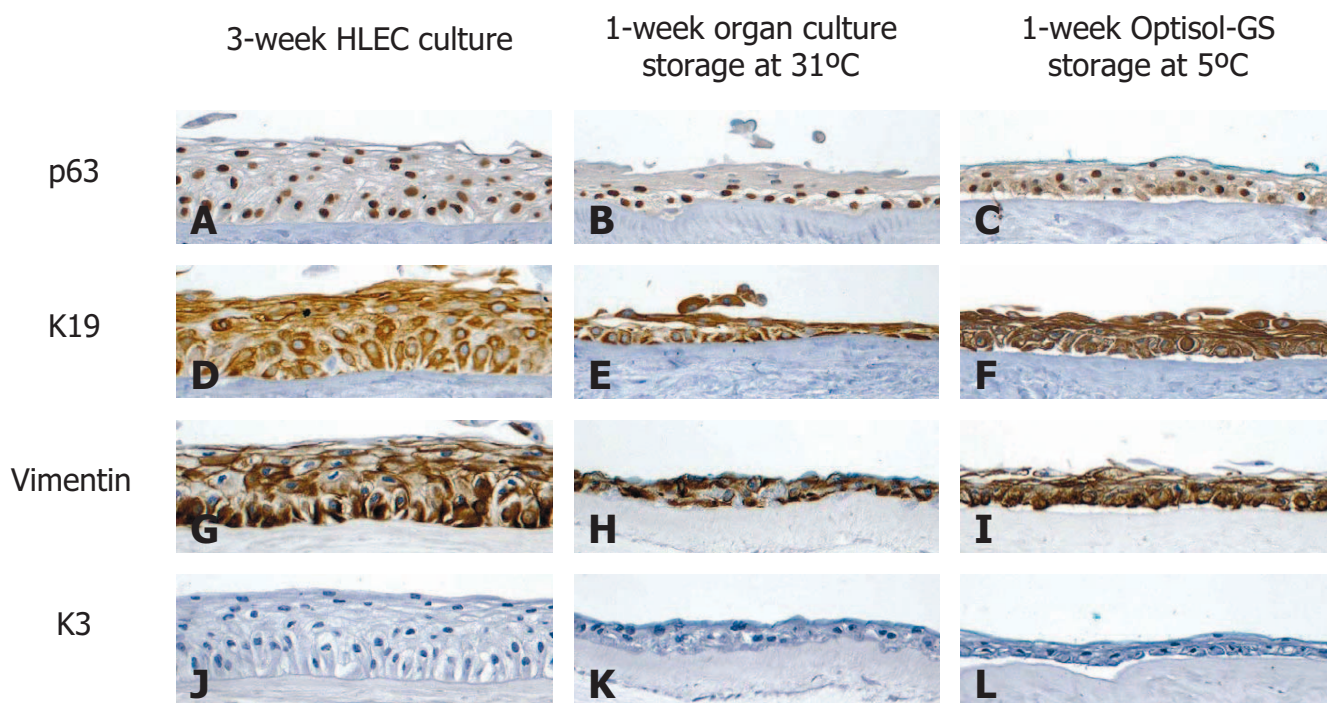


FIGURE 4. Immunostaining of p63 (A–C), K19 (D–F), vimentin (G–I), and K3 (J–L) in cultured human limbal epithelial cells after 3 weeks' culture and 1 week's storage at 31°C and 5°C. The expression of markers of undifferentiated cells (p63, K19, and vimentin) was maintained after 31°C OC storage and hypothermic preservation. The undifferentiated nature of the cells after eye bank storage was supported by the negative expression of K3, a marker of corneal epithelial differentiation. Original magnification, $\times 400$.

primers. The total volume of the reaction was 20 μ L diluted to 100 μ L. PCR reactions were performed using real-time PCR (79s00HT 384-well block with RT² Real-Time SYBR Green PCR master mix PA-012; Applied Biosystems). The total volume of the PCR reaction was 20 μ L. An equivalent of 0.4 ng of RNA was applied to the PCR reaction. The thermocycler parameters were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene expression of stored HLECs was compared with 3-week HLEC cultures. Relative changes in gene expression were calculated using the $\Delta\Delta C_t$ (cycle threshold) method.⁴² An average of the number of cycles of the five housekeeping genes, GAPDH, Actin- β , β 2m, Hprt1, and Rpl13d, was used to normalize the expression between samples. The expression data are presented as actual change multiples.

Cleaved Caspase-3 Immunohistochemistry and TUNEL Assays

Immunohistochemistry was performed as just described, with an antibody specific for cleaved caspase-3 (dilution 1:100). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed with a colorimetric TUNEL system according to the manufacturer's protocol (Promega). At $\times 400$ magnification, cells from the whole length of the epithelial outgrowth with condensed nuclei and positive labeling with anti-caspase-3 and TUNEL were counted as apoptotic by two independent experienced investigators. The apoptotic index, caspase-3 labeling index, and TUNEL labeling index were used as quantitative measures of apoptosis in histologic sections as previously reported by Duan et al.⁴³

Statistical Analysis

Statistical comparison of real-time PCR data was performed with the nonpaired Student's *t*-test (Excel; Microsoft, Redmond, WA) with 3-week HLEC cultures serving as control samples.

The apoptotic and labeling indices were tested against the respective indices in 3-week HLEC cultures by using the Mann-Whitney test

(SPSS ver.14.0; SPSS Inc., Chicago, IL). $P < 0.05$ was considered significant.

RESULTS

Epithelial Morphology

After storage at 31°C, extensive detachment of epithelial cells occurred (Fig. 2B). Fibroblasts were noted in three of eight replicates. Weak chromatin condensation was occasionally present, but clumping of nuclear chromatin and rupture of the cell membranes were not observed. The spaces between adjacent cells increased considerably (Fig. 3D). A few desmosomes, separation of desmosomes, and detachment of desmosome complexes were revealed. The basal cells were poorly attached to the amniotic membrane via a small number of hemidesmosomes. Intracellular vacuoles were common.

Storage of HLEC cultures at 23°C did not induce chromatin condensation, nuclear fragmentation, or clumping of nuclear chromatin, and cell membranes remained intact (Fig. 3E). Inter-cellular spaces increased slightly, and numerous desmosomal junctions were seen between adjacent superficial epithelial cells (Fig. 3F). The polymorphic basal cells attached well to the amniotic basement membrane by hemidesmosomes (Fig. 3G). Intracellular vacuoles were observed infrequently.

Storage of HLEC cultures in hypothermic conditions demonstrated considerable enlargement of intercellular spaces, separation of desmosomes, detachment of epithelial cells, detachment of the epithelia from the AM, and an increased number of intracellular vacuoles (Figs. 2C, 3H). In addition to weak to moderate chromatin condensation, rupture of cell membranes, and dissolution of organelles were regularly observed.

Three-week HLEC cultures served as control cultures and showed a multilayered epithelium (Fig. 3A) with numerous

TABLE 2. Up- or Downregulation of Anti- and Proapoptotic Genes in Cultured HLECs after 1-Week's Storage at Three Different Temperatures

Gene Symbol	GenBank Accession No.	1-Week Organ Culture Storage at 31°C	1-Week Organ Culture Storage at 23°C	1-Week Optisol-GS Storage at 5°C
Antiapoptotic genes				
<i>BAG4</i>	NM004874	4.51	7.65*	2.49
<i>BCL2</i>	NM000633	2.09	18.87*	12.56*
<i>BCL2A1</i>	NM004049	-4.14	-6.13*	-4.98*
<i>BIRC1</i>	NM004536	-3.62	-27.47*	-16.64*
<i>BIRC6</i>	NM016252	-1.86	5.20*	2.35
<i>BIRC8</i>	NM033341	-6.69*	-8.94*	-7.04*
<i>BNIP2</i>	NM004330	4.70*	11.61*	11.58*
<i>CARD6</i>	NM032587	1.43	6.14*	3.42*
<i>MCL1</i>	NM021960	-8.96*	-32.41*	-22.96*
Proapoptotic genes				
<i>ABL1</i>	NM005157	10.46*	4.22	2.54
<i>APAF1</i>	NM001160	2.16	4.88*	3.22
<i>BAK1</i>	NM001188	-7.44*	-4.39	-7.50*
<i>BCL2L11</i>	NM006538	2.08	8.72*	-3.20
<i>BNIP3L</i>	NM004331	5.54	13.27*	7.58*
<i>CARD4</i>	NM006092	3.64	5.00*	-1.16
<i>CARD8</i>	NM014959	4.97	30.16*	10.36*
<i>CASP5</i>	NM004347	-1.68	17.81*	11.53*
<i>CASP6</i>	NM032992	15.78*	15.21*	17.79*
<i>CASP9</i>	NM001229	-4.05*	-1.27	-2.03*
<i>CIDEB</i>	NM014430	10.21*	23.86*	11.75*
<i>FADD</i>	NM003824	-3.37	16.72*	10.80*
<i>FAS</i>	NM000043	1.71	4.93*	6.00
<i>FASLG</i>	NM000639	1.01	14.25*	6.93
<i>GADD45A</i>	NM001924	1.08	-12.23*	-8.85*
<i>HRK</i>	NM003806	-2.02	-3.01*	-3.13*
<i>NOL3</i>	NM003946	1.15	-3.86*	-4.51*
<i>PYCARD</i>	NM013258	-1.34	-5.67*	-2.27*
<i>RIPK2</i>	NM003821	-2.13	7.07	9.44*
<i>TNF</i>	NM000594	-3.17	-18.24*	-14.30*
<i>TNFRSF9</i>	NM001561	-4.67	-4.09*	-5.07*
<i>TNFRSF10</i>	NM003810	-9.32	-4.21*	-3.23
<i>TP53</i>	NM000546	1.30	-5.75*	-4.28*
<i>TRADD</i>	NM003789	-1.81	-9.11*	-4.25*

The expression data is presented as actual change multiples (\times -fold). Gene expression of stored HLECs was compared with that in 3-week HLEC cultures. Relative changes in gene expression were calculated using the $\Delta\Delta C_t$ (cycle threshold) method.⁴² An average of the cycle numbers of the five housekeeping genes *GAPDH*, *Actin-b*, *b2m*, *Hprt1*, and *Rpl13d* was used to normalize the expression between samples.

* Significant changes ($P < 0.05$) on the basis of reliable C_t values.

intercellular desmosomes (Fig. 3B) and hemidesmosomes (Fig. 3C).

Phenotypic Characterization

The cultured HLECs remained undifferentiated (p63-, K19-, and vimentin-positive and K3-negative) under 31°C OC storage and hypothermic storage conditions (Table 1, Fig. 4).

Apoptosis Gene Expression Profiling

Table 2 shows the anti- and proapoptotic genes in cultured HLECs after 1 week's storage at three different temperatures. Expression of DNA fragmentation factor (DFFA) was not significantly altered in stored HLECs, whereas the expression of caspase-3 was below the level of detection. After storage at 23°C and 5°C, upregulation of *BCL2*, downregulation of *BCL2A1* and *BIRC1*, and reduced expression of *TNF* receptor signaling components (*TNF* and *TRADD*) were revealed. Furthermore, upregulation of components of the Fas-mediated pathway (*FAS*, *FASLG*, and *FADD*) and *BAG4* and downregulation of *PYCARD* were observed in 23°C storage conditions. In all storage conditions, the expression of *BNIP2* was upregulated, whereas the expression of *MCL1* was downregulated.

Quantification of Apoptotic Cells

Few apoptotic cells were observed in all storage conditions (Table 3, Figs. 5, 6) giving low labeling indices of caspase-3 (range, 0.0%–4.7%) and TUNEL (range, 0.0%–7.8%). When comparing the experimental groups with the control group, there was a trend toward a higher apoptotic index with decreased storage temperature, although the differences were not statistically significant.

DISCUSSION

In the present study, conventional OC storage at 31°C and hypothermic eye bank culture were clearly inferior to the 23°C OC preservation method in preserving the original layered structure of cultured HLECs. Eye bank storage of cultured HLECs was associated with minor phenotypic changes and limited cell death due to apoptosis in all three storage conditions.

Detachment of epithelial cells was observed consistently after storage at 31°C and 5°C, in sharp contrast to storage at 23°C, where there was no sign of detachment. The morphologic characteristics of cultured HLECs stored at 31°C are in

TABLE 3. Apoptotic Index, Caspase-3 Labeling Index, and TUNEL Labeling Index in Cultured HLECs after 3 Weeks' Culture and 1 Week's Storage at Three Different Temperatures

Group	<i>n</i>	Mean	SD	Maximum	Percentage of Samples with Index = 0	<i>P</i> *
H&E apoptotic index (%)†						
3-Weeks HLEC culture	8	0.1	0.2	0.6	75.0	—
1-Week OC storage at 31°C	7‡	0.1	0.3	0.7	85.7	0.87
1-Week OC storage at 23°C	8	0.1	0.2	0.6	62.5	0.88
1-Week Optisol-GS storage at 5°C	8	0.3	0.8	2.3	87.5	0.80
Caspase-3 labeling index (%)§						
3-weeks HLEC culture	8	0.1	0.2	0.5	87.5	—
1-week OC storage at 31°C	7‡	0.3	0.6	1.6	71.4	0.54
1-week OC storage at 23°C	8	0.2	0.3	0.9	50.0	0.28
1-week Optisol-GS storage at 5°C	8	1.2	1.8	4.7	50.0	0.20
TUNEL labeling index (%)						
3-Weeks HLEC culture	8	0.2	0.6	1.6	87.5	—
1-Week OC storage at 31°C	7‡	1.0	1.7	4.8	42.9	0.19
1-Week OC storage at 23°C	8	1.2	1.6	3.7	50.0	0.20
1-Week Optisol-GS storage at 5°C	8	2.3	2.8	7.8	37.5	0.08

* Calculated by testing the labeling index of the individual experimental group against the labeling index of 3-week HLEC culture.

† H&E apoptotic index = number of apoptotic cells (condensed nuclei) × 100/total number of nuclei.

‡ One of eight samples was excluded from the analysis because of extensive ingrowth of fibroblasts.

§ Caspase-3 labeling index = Number of activated caspase-3-positive cells × 100/total number of nuclei.

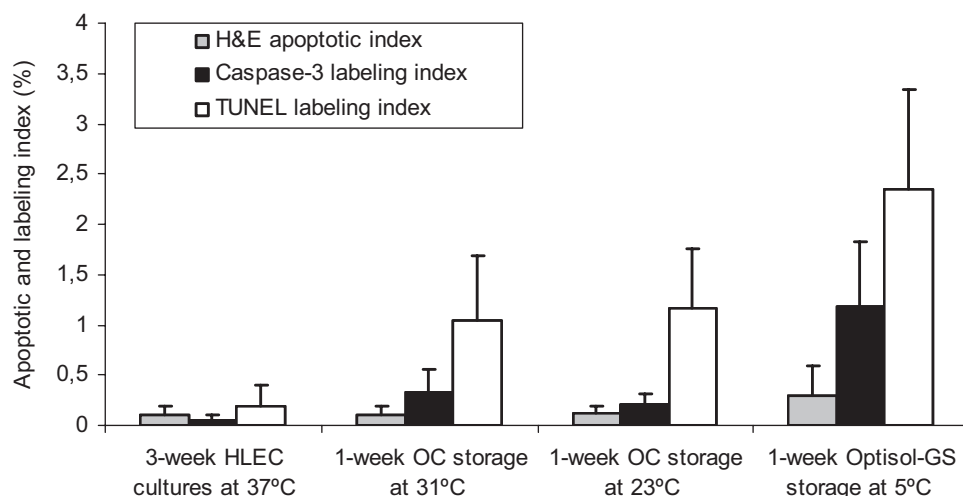
|| TUNEL labeling index = number of TUNEL-positive cells × 100/total number of nuclei.

line with those observed in studies of organ-cultured cornea performed at 31°C, which describe epithelial sloughing of two to three cell layers after 7 days⁴⁴ and intracellular vacuoles.^{44,45} Reduction of epithelial thickness has also been registered after OC storage of corneas at 37°C^{46,47} and 34°C.³⁶ Furthermore, studies of organ-cultured corneas at 37°C have reported dilated intercellular spaces^{46,47} and a decreased number of desmosomes,⁴⁶ both of which are consistent with our findings. With regard to storage at 5°C, morphologic findings similar to those in the present study were found in a study of human corneas stored for 6 to 10 days in Optisol-GS, demonstrating pronounced intracellular edema and separation of the cells below the superficial layer.⁴⁸

Although no specific marker for the limbal epithelial stem cell has been identified to date, the description of an undifferentiated limbal epithelial phenotype currently relies on the combination of positive expression of putative stem cell-associated markers and negative or low staining of differentiation-

associated markers. In the present study, the transcription factor p63 and the cytoskeletal proteins K19 and vimentin were expressed after all storage conditions. Previous studies have shown that p63 is expressed in corneal epithelial cells with high proliferative capacity, denoted transient amplifying cells (TACs).^{49–51} K19 and vimentin are localized to the basal cells of the limbal epithelium and have been suggested as stem cell candidate markers^{40,52,53}; however, a later study demonstrated that K19 was also expressed by corneal epithelial cells.⁴¹ The undifferentiated nature of the cells after eye bank storage was supported by the negative expression of K3, a marker of corneal epithelial differentiation.⁵⁴

The positive expression of the gap junction protein Cx43 in our study is consistent with a recent investigation by Chen et al.⁵⁵ who reported that 60% of cultured HLECs expressed Cx43. However, previous reports have demonstrated that Cx43 is expressed in the suprabasal layer of limbal epithelium and suggested that Cx43 expression represents differentiation

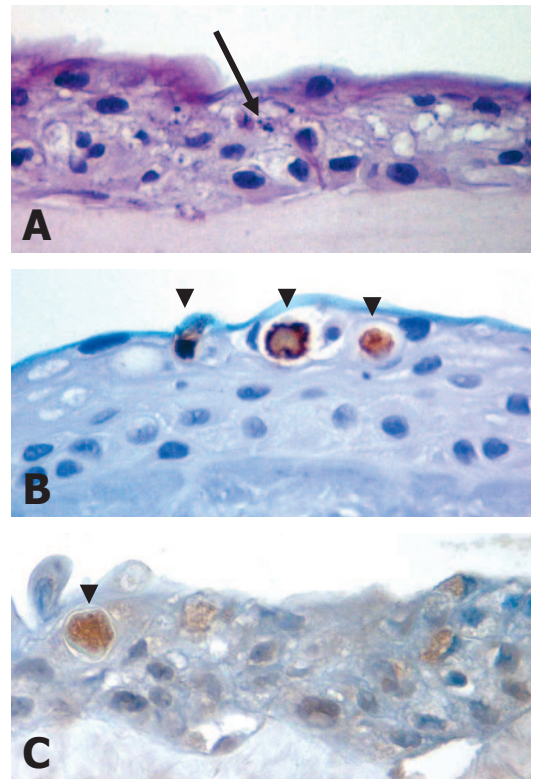
**FIGURE 5.** Histogram illustrating the H&E apoptotic index, caspase-3 labeling index, and TUNEL labeling index in cultured HLECs after 3 weeks' culture and 1 week's storage at three different temperatures. Results are expressed as the mean percentage of the apoptotic or labeling index in the individual experimental groups. Error bars, 1 SE.

H&E

Cleaved caspase-3

TUNEL

FIGURE 6. H&E staining, cleaved caspase-3 immunohistochemistry, and TUNEL staining of cultured human limbal epithelial cells after 1 week's organ culture storage at 23°C. (A) H&E staining demonstrating an apoptotic epithelial cell with circular nuclear fragments (*arrow*). (B) Cleaved caspase-3-positive surface cells with cytoplasmic immunoreactivity and well-defined nuclear membranes (*arrowheads*). (C) TUNEL-positive surface cell (*arrowhead*). Original magnification, $\times 400$.



of corneal TACs.^{41,56,57} Furthermore, Hernandez Galindo et al.⁵⁰ have suggested that the coexpression of delta p63 (clone 4A4) and Cx43 in HLEC cultures may indicate early TACs. Positive expression of the keratin pair K5/K14 and integrin $\beta 1$ may also be indicative of TAC differentiation as limbal and corneal basal cells are shown to express these markers.^{41,51,58,59}

The immunohistochemical analyses may also provide insight into cell survival after eye bank storage of cultured HLECs. Maintenance of high p63 expression and minimal changes in expression of Ki67, a proliferating cell nuclear marker, suggest that eye bank storage preserves the proliferative capacity of cultured HLECs. Furthermore, the expression of the transmembrane receptor E-cadherin was sustained in most groups and has been reported to facilitate cell proliferation and survival.⁶⁰

Intercellular edema may give an explanation of the considerable cell detachment under 31°C and 5°C storage conditions. However, cell death due to apoptosis^{61,62} has been reported in human corneal epithelium after OC³⁶ and hypothermic^{37,38} storage. In the present study, signs of chromatin condensation were revealed under 31°C and 5°C storage conditions. Accordingly, we postulated that apoptosis might contribute to the detachment of epithelial cells; however, immunohistochemistry for cleaved caspase-3 and TUNEL showed no significant increase in response to eye bank storage.

Multigene profiling revealed interesting alterations in gene expression in cultured HLECs after eye bank storage. Several of the changes in gene expression in cultured HLECs under 23°C and 5°C storage conditions suggested a reduction in nuclear factor (NF)- κ B activity, inasmuch as several apoptosis-regulating genes that are NF- κ B targets were reduced in their expression, including *BCL2A1*, *BIRC1*, *TNF*, and *PYCARD*. TNF receptor adapter protein, TRADD, was also reduced, whereas expression of BAG4, an antagonist of TNF receptor signaling, was increased. Furthermore, expression of CARD6, a modulator of certain NF- κ B activation pathways,⁶³ was increased.

NF- κ B protein is one of the major transcription factors.^{64,65} The activation of NF- κ B leads to synthesis of proinflammatory cytokines, including TNF- α and IL-1 β , which mediate inflammatory and immune responses⁶⁶ and protect the cells from undergoing apoptosis.^{67–69} Investigations at protein levels leading to clinical studies are warranted to elucidate the importance of a reduction in NF- κ B activity to answer whether eye bank storage of cultured HLECs lessens immunoreaction and reduces the need for immunosuppression after transplantation.

Components (*FAS*, *FASLG*, and *FADD*) of the extrinsic pathway for cell death and caspase activation^{70–72} were all prominently upregulated in 23°C storage conditions. Furthermore, expression of *MCL1*, an antiapoptotic gene belonging to the *BCL2* family, was profoundly downregulated, and expression of the *BCL2* antagonists *BNIP2* and *BNIP3L* was increased. It remains to be determined why these changes in gene expression were not associated with increased apoptosis. Downstream blocks to Fas-mediated apoptosis, including upregulation of the NF- κ B-inducible antiapoptotic proteins, BAG4 and CARD6, may have neutralized the increased expression of Fas-pathway components. In addition, the intrinsic pathway for caspase activation^{73,74} may have been inhibited by the strong upregulation of *BCL2*, an inhibitor of apoptosis acting upstream of the activation of caspase in mitochondrial and endoplasmic reticulum pathways for cell death.⁷⁵ In support of the final supposition, *BCL2* is suggested to modulate apoptotic cell desquamation in the human corneal epithelium.⁷⁶

There are several issues to be resolved in eye bank storage of cultured HLECs. First, further studies are needed to explore the biological mechanisms underlying the morphologic changes in cultured HLECs after eye bank storage at different temperatures. Second, studies are warranted on prevention of cell detachment during eye bank storage. The use of epithelially denuded AM and air-lifting has been reported to increase the number of desmosomal junctions and decrease intercellular spaces.^{16,22} Furthermore, air-liquid corneal organ culture

has been described to decrease epithelial intercellular edema.⁷⁷ Finally, the effects of the various HLEC culture protocols on eye bank storage of cultured corneal epithelium should be pursued in future studies, including cell suspension culture, serum-free culture, and storage medium- and carrier-free techniques.

In conclusion, our data indicate that OC storage of cultured HLECs at ambient temperature is superior to OC storage at 31°C and Optisol-GS storage at 5°C, and that apoptosis is minimal after eye bank storage of cultured HLECs. We believe that eye bank storage of cultured HLECs may provide a reliable source of tissue for treating limbal stem cell deficiency, although its feasibility for clinical use should be evaluated further.

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